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ORIGINAL PAPER

The CphAII protein from *Aquifex aeolicus* exhibits a metal-dependent phosphodiesterase activity

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Abstract The CphAII protein from the hyperthermophile *Aquifex aeolicus* shows the five conserved motifs of the metallo-β-lactamase (MBL) superfamily and presents 28% identity with the *Aeromonas hydrophila* subclass B2 CphA MBL. The gene encoding CphAII was amplified by PCR from the *A. aeolicus* genomic DNA and overexpressed in *Escherichia coli* using a pLex-based expression system. The recombinant CphAII protein was purified by a combination of heating (to denature *E. coli* proteins) and two steps of immobilized metal affinity chromatography. The purified enzyme preparation did not exhibit a β-lactamase activity but showed a metal-dependent phosphodiesterase activity versus bis-*p*-nitrophenyl phosphate and thymidine 5′-monophosphate *p*-nitrophenyl ester, with an optimum at 85°C. The circular dichroism spectrum was in agreement with the percentage of secondary structures characteristic of the MBL $\alpha\beta\beta\alpha$ fold.

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Abbreviations

MBL	metallo-β-lactamase
pNPS	*p*-nitrophenyl sulfate
pNPP	*p*-nitrophenyl phosphate
bpNPP	bis-*p*-nitrophenyl phosphate
TpNPP	*p*-nitrophenyl 5′-thymidine monophosphate
pNPPC	*p*-nitrophenylphosphorylcholine

Introduction

The metallo-β-lactamase (MBL) superfamily was defined in 1997 (Neuwald et al. 1997). In addition to MBLs which cleave the amide bond of the β-lactam ring of penicillins, cephalosporins or carbapenems thus inactivating the antibiotic (Bebrone 2007), the MBL superfamily includes enzymes which hydrolyze thiol-ester, phosphodiester and sulfuric ester bonds as well as oxidoreductases. The number of proteins identified as presenting the five MBL superfamily conserved motifs is rapidly increasing (currently, there are more than 18,500). These enzymes, classified in 17 families (Daiyasu et al. 2001), catalyze a variety of diverse reactions. Only 9 of the 17 protein groups proposed to contain a MBL $\alpha\beta\beta\alpha$ fold have been characterized structurally: class B β-lactamases also called MBLs (group 1) (Carfi et al. 1995), glyoxalases II (group 2) (Cameron et al. 1999), rubredoxin oxidoreductases (group 3) (Frazao et al. 2000), the phosphorylcholine esterase Pce (group 9) (Garau et al. 2005), the methyl parathion hydrolase (group 15) (Dong et al. 2005), the *N*-acyl

homoserine lactone hydrolase (group 12) (Liu et al. 2005), the alkylsulfatase from *Pseudomonas aeruginosa* SdsA1 (group 13) (Hagelueken et al. 2006), the tRNA3′-processing endoribonuclease tRNaseZ (group 4) (Ishii et al. 2005) and proteins members of the β-CASP family (groups 6 and 7) (Callebaut et al. 2002). Whereas β-lactamases were shown to be active with one or two zinc ions (Bebrone 2007), active glyoxalase II requires two of them (Cameron et al. 1999) or a mixed zinc/iron binuclear active site (Wenzel et al. 2004) and rubredoxin:oxygen oxidoreductase contains two iron ions (Frazao et al. 2000). The structural and biochemical characterization of this emerging MBL superfamily is necessary. Indeed, a large number of hypothetical enzymes found in GenBank™ might also share this $\alpha\beta\beta\alpha$ fold and bind one or two metal ions, but most of the enzymes have not been functionally or structurally characterized yet. One of these hypothetical MBL proteins is the product of the *cphAII* gene in *Aquifex aeolicus* VF5 (GenBank accession number AE000657, locus_tag = "aq_974"). *A. aeolicus* is a chemolithoautotrophic bacterium that thrives at temperatures higher than 85°C in environments containing only inorganic components and utilizes gaseous hydrogen, carbon dioxide and oxygen as substrates (Huber et al. 1992; Kawasumi et al. 1984). The complete genomic sequence of an *A. aeolicus* strain isolated from hydrothermal vents in Yellowstone National Park was determined by Deckert et al. (1998). The *A. aeolicus* CphAII protein sequence shows the five conserved motifs of the MBL superfamily, namely Asp84, His116-Xaa-His118-Xaa-Asp120-His121, His196, Asp221 and His263 [the standard numbering for class B β-lactamases (BBL) (Garau et al. 2004) is applied to CphAII and used throughout this paper)] (Fig. 1a). With the exception of Asp84, the other conserved residues are involved in metal coordination. Indeed, for the majority of the already characterized enzymes of the superfamily, the first metal ion-binding site is composed of His116, His118 and His 196; Asp120, His121 and His263 form the second one. Asp221 and a water molecule bridge the two metallic ions (Bebrone 2007).

CphAII (O67103_AQUAE) is annotated as a β-lactamase precursor in the Protein Knowledgebase UniProtKB. It presents 28% identity with the *Aeromonas hydrophila* subclass B2 CphA MBL (Hernandez-Valladares et al. 1996, 1997; Vanhove et al. 2003; Bebrone et al. 2005, 2008, 2009) (Fig. 1b) and 25% identity with the *Pseudomonas aeruginosa* subclass B1 SPM-1 MBL (Murphy et al. 2006). The percentage of identity between the three subclasses of "real" MBLs (B1, B2 and B3) also does not exceed 30%. However, a major difference with "real" MBLs is that the CphAII sequence highlights the presence of an Asp residue in position 221 as in most members of the superfamily, whereas a cysteine is found in B1 and B2 β-lactamases and a serine (or methionine in GOB-type enzymes) in B3 (Fig. 1a). No enzymatic property was determined for the product of the *cphA*II gene from *A. aeolicus*. We produced this protein in *Escherichia coli* and purified it to homogeneity. We show here that this protein is not a β-lactamase, but exhibits a metal-dependent phosphodiesterase activity versus bis-*p*-nitrophenyl phosphate (bpNPP) and thymidine 5′-monophosphate *p*-nitrophenyl ester (TpNPP), two small artificial chromogenic substrates.

Experimental

Bacterial strains and genetic materials

E. coli DH5α was used as the host for recombinant plasmids during plasmid construction. *E.coli* GI724 (Invitrogen) was used as the host for expression of the CphAII enzyme cloned in the pLex expression vector. pGEM T-easy vector (Promega) was used as the vector for cloning and confirmatory sequencing of the PCR-amplified *cphA*II gene. pLex (Invitrogen) and pLex-His vectors were used as the expression vectors for the production of the CphAII enzyme. This latter vector was a gift from Dr. A. Chahboune (CIP, University of Liège, Belgium) and was constructed as a modified pLex vector where the polylinker of pLex is replaced by the polylinker of the pET22b vector (Novagen). *A. aeolicus* VF5 genomic DNA was a gift from Dr. M. Ngyen-Distèche (CIP, University of Liège, Belgium).

Cloning, expression and purification of CphAII

To develop an expression system for overproduction of the CphAII enzyme, the *A. aeolicus cphA*II gene was cloned in the pLex-His vector as follows. The *cphAII* ORF was amplified by PCR from the *A. aeolicus* genomic DNA using primers 5′-GGTTCTAACTTTCATATGGGAGGTG CGGTCATGTTAAAAACGC-3′ and 5′-CTCGAGAAGT TCTAAATCAAGTTCGTTGTAAACCTTCCACACGTT TGT-3′ to obtain an amplification product containing the entire *cphA*II ORF flanked by restriction sites (*Nde*I and *Xho*I), suitable for directional cloning in the polylinker of the pLex-His vector, downstream of the strong and highly regulated P_L promoter carried by this vector. The PCR was performed with *Taq* polymerase (Promega, Heidelberg, Germany). Reaction conditions were as follows: denaturation for 1 min at 95°C, annealing for 1 min at 63°C and extension for 2 min at 72°C, repeated for 30 cycles. The amplification product was initially cloned in the pGEM vector, giving pGEM/CphAII, and subjected to sequencing

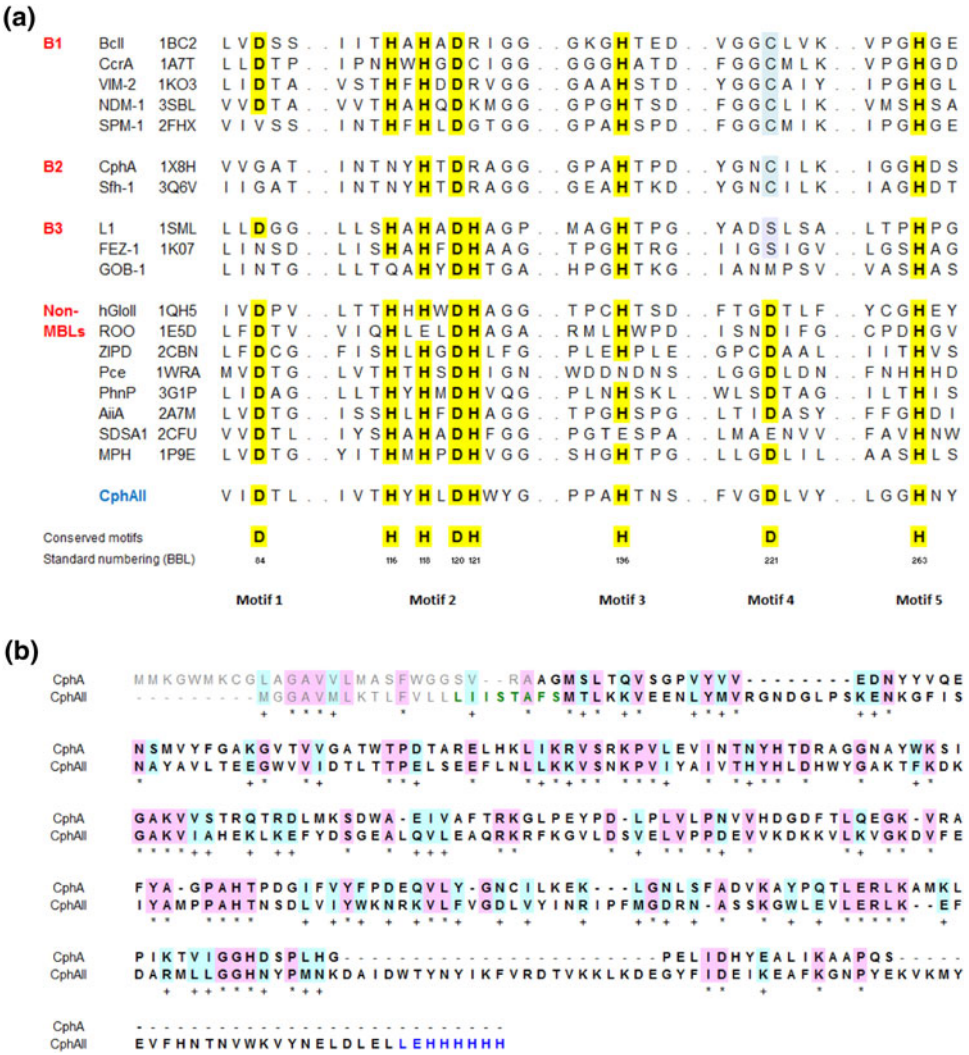

Fig. 1 **a** Multiple alignment of representative MBL superfamily members, highlighting the five conserved motifs. MBLs from *Bacillus cereus* (BcII), *Bacteroides fragilis* (CcrA), *Pseudomonas aeruginosa* (VIM-2, SPM-1), *Klebsiella pneumonia* (NDM-1), *Aeromonas hydrophila* (CphA), *Serratia fonticola* (Sfh-1), *Stenotrophomonas maltophilia* (L1), *Legionella gormanii* (FEZ-1), *Elizabethkingia meningoseptica* (GOB-1). Non-MBLs superfamily members: human glyoxalase II (hGloII), rubredoxin:oxygen oxidoreductase from *Desulfovibrio gigas* (ROO), zinc-dependent phosphodiesterase from *Escherichia coli* (ZIPD), phosphorylcholine esterase from *Streptococcus pneumonia*, phosphodiesterase of the carbon-phosphorus lyase pathway from *E. coli* (PhnP), acyl-homoserine-lactone lactonase from *Bacillus thuringensis* (AiiA), alkylsulfatase from *P. aeruginosa* (SDSA1) and methylparathion hydrolase from *Pseudomonas* sp. (MPH). Available Protein data Bank accession codes are indicated. **b** Sequence comparison between CphAII from *Aquifex aeolicus* and the subclass B2 CphA β-lactamase from *Aeromonas hydrophila*. The percentage of sequence identity is 28%. The mature sequences are in *bold black* while the sequences of the signal peptides are in *gray*. The eight additional amino-acids at the N-terminal of CphAII when compared to the theoretical N-terminal sequence are in *green*. Identical residues are highlighted in *pink* and similar residues in *cyan*. The LEHHHHHH peptide was added at the C-terminal as a His-Tag to facilitate the purification (residues in *blue*)

to verify that the in vitro PCR step had not introduced unwanted mutations. The ~900 bp *Nde*I–*Xho*I insert of pGEM/CphAII was then subcloned in the pLex-His vector to obtain plasmid pLex/CphAII-His.

The GI724 *E. coli* cells harboring pLex/CphAII-His were grown overnight at 30°C in 60 ml of RM medium (per liter: 20 g casamino-acids, 3 g KH_2PO_4, 0.5 g NaCl, 6 g Na_2HPO_4, 1 g NH_4Cl, 0.095 g $MgCl_2$, 1% (v/v) glycerol) containing 100 μg ml^{-1} ampicillin as the selection agent. This initial culture was added to 2 l of induction medium (per liter: 2 g casamino-acids, 3 g KH_2PO_4, 0.5 g NaCl, 6 g Na_2HPO_4, 1 g NH_4Cl, 0.095 g $MgCl_2$, 5 g glucose) containing 100 μg ml^{-1} ampicillin. The culture was grown at 30°C. At an A_{550} value of 0.5, 20 ml of 10 mg ml^{-1} tryptophan was added and the culture grown for a further 20 h.

The bacteria were harvested by a 15-min centrifugation at 5,000×*g* at 4°C. The pellet was resuspended in 60 ml of 50 mM sodium phosphate buffer pH 8.0, containing 250 mM NaCl (buffer A1) and subjected to cellular

disruption (EmulsiFlex C5, Avestin, Mannheim, Germany). Cell lysates were then centrifuged at 30,000×*g* for 30 min at 4°C. The resulting supernatant was heated at 80°C for 10 min to denature thermolabile *E. coli* proteins and placed on ice for 30 min to aggregate the denatured proteins. The supernatant was separated from the precipitated proteins by centrifugation at 30,000×*g* for 30 min at 4°C. The cleared supernatant was dialyzed overnight at 4°C against 5 l of buffer A1 and then loaded onto a 5 ml HisTrap™ HP (Amersham Biosciences) equilibrated with the same buffer. The column was washed with buffer A1 and the protein eluted with an imidazole gradient (0–300 mM). The fractions containing CphAII were collected, pooled, diluted sixfold in buffer A1 and then loaded onto a 1 ml HisTrap™ HP column (Amersham Biosciences) equilibrated with the same buffer. The column was washed with buffer A1 and eluted with an imidazole gradient (0–300 mM). The fractions containing CphAII were collected, pooled, dialyzed against buffer A1 and concentrated by ultrafiltration with an Amicon ultrafiltration apparatus using an YM-10 membrane. The protein sample was concentrated to 1 mg ml^{-1}, as determined by the Bradford assay (Fermentas) or by measuring the absorbance at 280 nm ($A_{280}^{1\ mg/ml} = 1.48$). 0.02% (w/v) sodium azide was added to the protein solution. No activity was lost after prolonged storage at 4°C (several months).

To evaluate the influence of the C-terminal His-Tag on the zinc binding, CphAII was also produced without this added His-Tag. To do so, *cphAII* was cloned using primers (5′-GGTTCTAACTTTCATATGGGAGGTGCGGTCATG TTAAAAACGC-3′ and 5′-CCATAACGTGGATCCTCA AAGTTCTAAATCAAGTTCGTTGTA-3′) introducing restriction sites for *Nde*I and *BamH*I, respectively. PCR conditions and sub-cloning in pGEM were as described above. The gene coding for CphAII was then cloned into the *Nde*I and *BamH*I sites of the original pLex vector, yielding pLex/CphAII. Production of the CphAII protein was obtained from GI724 *E. coli* cells harboring the pLex/CphAII vector, following the protocol described above. CphAII without added His-Tag was purified by a combination of heating and ion-exchange chromatography. Briefly, the bacterial suspension was pelleted, resuspended in 60 ml of MES 10 mM pH 5.5 (buffer A2) and then disrupted and clarified by centrifugation. The resulting supernatant was heated at 80°C for 10 min and then placed on ice for 30 min. After centrifugation at 30,000×*g* for 30 min at 4°C, the supernatant was dialyzed against buffer A2, loaded onto an SP-Sepharose FF column pre-equilibrated with the same buffer and the CphAII protein was eluted with a linear NaCl gradient (0–0.5 M). Fractions containing CphAII were pooled, concentrated to 1 mg ml^{-1} and dialyzed overnight against buffer A1 at 4°C.

Gel filtration

To determine whether purified CphAII was in mono- or oligomeric form and to determine its molecular mass, the protein was loaded onto a Superdex 75 GL 10/300 column previously calibrated with insulin (5,800 Da), chicken lysozyme (14,300 Da), trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), chicken egg ovalbumin (45,000 Da), bovine albumin (66,200 Da) and dextran blue (2,000,000 Da) as molecular mass standards. The flow rate of buffer A was 0.2 ml min^{-1}. Approximately, 200 μg of CphAII in 0.2 ml was applied to the column.

Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the protocol of Laemmli (1970) with 5% stacking and 15% resolving gels using a Mini Protean III Biorad system. Protein bands were stained with Coomassie brilliant blue R-250 (Fluka, Buchs, Switzerland), and the molecular mass of the purified CphAII was determined by comparing its relative mobility to those of standard protein markers (Unstained Protein Molecular Weight Marker, Fermentas).

Determination of the total-mass by mass spectrometry

The mass of CphAII was determined by the ESI MS mass spectrometric method (Micromass Electrospray Q-Tof-2, Waters Corporation, MA, USA). The deconvolution was performed with the Maxent 1, Masslynx 4.0 program (Waters Corporation, MA, USA).

Protein identification

The N-terminal sequence of the purified protein was determined with the help of a gas-phase sequencer (Prosite 492 prosite sequencer, Applied Biosystems, Forster City, California).

Furthermore, the identity of CphAII was verified using peptides from a tryptic digestion analyzed with a nano-HPLC (Dionex, CA, USA) coupled to ESI–MS/MS mass spectrometer (Micromass Electrospray Q-Tof-2, Waters Corporation, MA, USA). Protein identification was carried out using the search engine MASCOT (Matrix Science, Boston, MA, USA).

Circular dichroïsm

Circular dichroism spectra were recorded using a J-810 CD spectropolarimeter (Jasco). The spectra were scanned at 25°C with 1 nm steps from 185 to 260 nm (far-UV). 37 μg of purified CphAII was used in 300 μl of 10 mM sodium

phosphate buffer pH 8.0 in a 1-mm quartz cuvette. Deconvolution of the CD spectra was performed using the CDSSTR program and the protein data sets no. 4 and 7, found in DICHROWEB, a facility of the Biotechnology and Biological Sciences Research Council Centre for Protein and Membrane Structure and Dynamics (Whitmore and Wallace 2008). The thermal stability of CphAII was assessed by increasing the temperature from 25 to 95°C and monitoring the ellipticity at 220 nm.

Determination of the zinc content by inductively coupled plasma mass spectrometry (ICP/MS)

Three-milliliter samples of CphAII (15 μM) were dialyzed against 50 mM sodium phosphate buffer pH 8.0 prepared with MilliQ water ([Zinc] < 0.4 μM) or with 100 μM zinc. An aliquot of the final dialysis buffer was saved for ICP/MS analysis of background zinc ion content. Protein concentrations were determined by the Bradford assay (Fermentas) or by measuring the absorbance at 280 nm. Zinc concentrations were measured by inductively coupled plasma mass spectroscopy (ICP/MS) at the Malvoz Institute (Liège, Belgium). The metal/enzyme ratio was calculated from the differences of metal concentration between the enzyme sample and the dialysis buffer. The enzyme preparation was also analyzed for the presence of some other divalent metals (cobalt, copper, manganese, nickel and iron).

Activity screening

General enzymatic screens for various activities (phosphatase, phosphodiesterase, esterase, protease, dehydrogenase and oxidase) were performed in microplates at 21 and 70°C as previously described (Miller et al. 2007; Kuznetsova et al. 2005; Chen et al. 2004; Yakunin et al. 2004). Moreover, several substrates of proteins which belong to the MBL superfamily were tested. Activity tests were performed in microplates in 150 μl of buffer A1. The color change from yellow to red due to the hydrolysis of nitrocefin (100 μM), a chromogenic β-lactamase substrate, was detected by eye. Carbapenemase and penicillinase activities using the substrates imipenem (100 μM) and ampicillin (1 mM) were monitored at 300 and 235 nm, respectively, using a Power Wave X (Bio-Tek instruments, INC.) microplate spectrophotometer. Liberation of *p*-nitrophenol from *p*-nitrophenyl sulphate (pNPS), *p*-nitrophenyl phosphate (pNPP), bis-*p*-nitrophenyl phosphate (bpNPP) or thymidine 5′-monophosphate *p*-nitrophenyl ester (TpNPP) (10 mM) was eye-detected by appearance of yellow color while the hydrolysis of *p*-nitrocatechol sulfate (10 mM) was eye-detected by a color change from yellow to red. Glyoxalase II activity using the substrate *S*-D-lactoylglutathione (0.5 mM) was monitored at 240 nm using a Power Wave X microplate spectrophotometer. All reactions were carried out at 21°C and monitored for 24 h. The reactions were also performed at 85°C. Negative controls were conducted without added enzyme. The alkylsulfatase activity of CphAII was also tested against the standard substrate rac-octyl-2-sulfate.

For phosphodiesterases, there are three main groups of natural substrates: cyclic nucleotides, nucleic acids and phospholipids. Phosphohydrolase activities against these substrates were then assayed using published protocols (Vogel et al. 2002; Chen et al. 2004; Podzelinska et al. 2009) and commercially available compounds. In brief, hydrolysis of DNA (single- and double-stranded) was inspected by analyzing reaction products by agarose gel electrophoresis. Hydrolysis of yeast RNA was analyzed by following the absorption change at 260 nm for 30 min after addition of CphAII. Hydrolysis of cyclic nucleotides (2′-3′-cAMP, 2′-3′-cGMP, 3′-5′-cCMP) was probed using Sigma alkaline phosphatase and malachite green reagent to detect the released inorganic phosphate. Reactivity with pyrophosphate, AMP, ADP, ATP was investigated by detection of released phosphate using molybdate/malachite green reagent. Phospholipase C activity was analyzed using 10 mM *p*-nitrophenylphosphorylcholine (pNPPC). Release of *p*-nitrophenolate was monitored at 405 nm. Phospholipase D activity against phosphatidylcholine was probed using choline oxidase (Sigma) and the peroxidase dianisine-coupled assay for oxidase testing (Kelley and Reddy 1988).

Phosphodiesterase assay for kinetic analysis

The hydrolysis of the chromogenic phosphodiesterase substrate (bpNPP or TpNPP) was monitored by following the absorbance variation at 405 nm, using an Uvikon 860 spectrophotometer equipped with thermostatically controlled cells and connected to a microcomputer.

Optimum temperature for the activity of the purified protein sample was determined by measuring the initial hydrolysis rates over the temperature range of 20–90°C with 10 mM bpNPP as the substrate. The exact temperature in the cell was verified with the help of a thermocouple.

The effect of pH and buffers on the activity versus 10 mM bpNPP was studied by measuring the initial hydrolysis rates at 85°C. The effect of pH and buffers on the stability of the enzyme was also investigated. The following buffers were used (the concentration of the buffering component was 50 mM in all cases): sodium acetate (pH 5, 5.5, 6), KH_2PO_4 (pH 6), sodium cacodylate (pH 6, 6.5), HEPES (pH 7, 7.5, 8), Tris (pH 7.4, 8 and 8.5) and sodium phosphate buffer (pH 7, 7.5 and 8) with or without 250 mM NaCl. The stability of CphAII at 50 and 85°C was also evaluated by measuring the activity of the

enzyme versus 10 mM bpNPP after different incubation times at these two temperatures.

The k_{cat} and K_m parameters were determined under initial-rate conditions, using the Hanes linearization of the Henri–Michaelis–Menten equation. The kinetic parameters (k_{cat}, K_m, k_{cat}/K_m) were determined in buffer A1 at two different temperatures (21 and 85°C).

Enzymatic measurement in the presence of increasing concentrations of cobalt or zinc and determination of K_{D2}

Relative activity in the presence of increasing concentrations of cobalt or zinc was measured at 85°C in buffer A1. Both metal ions are soluble in the phosphate buffer at least up to the highest tested concentration (500 μM). Binding of the second metal ion resulted in an increase of activity and Equation 1 was used:

$$RA = [(K_{D2} + \alpha[M])/([M] + K_{D2})] \times 100 \quad (1)$$

where RA is the relative activity and K_{D2} the dissociation constant for the second metal ion and α represents the ratio of activity at saturating metal concentration versus activity in the absence of added metal ($Act.[M]_{(\infty)}/Act.[M]_{(0)}$).

Experimental data were fitted to Eq. 1 by non-linear regression analysis with the help of the Sigma Plot software.

Results

Production of the *A. aeolicus* CphAII protein in *E. coli*

E. coli GI724 were transformed by plasmid pLex/CphAII-His and the production of the CphAII protein by the transformed strain was monitored as a function of time after induction by tryptophan at 37, 30 or 18°C. Results showed that the maximal production was achieved after 20 h of growth at 30°C. In these conditions, expression of CphAII in fusion with a C-terminal His-Tag in *E. coli* yielded ~5 mg/l of soluble recombinant protein. Assays to improve the quantity of produced recombinant CphAII protein with the help of various surexpression pET systems (pET9a, pET26b, pET28a from Novagen) and *E. coli* host strains (BL21(DE3), C41 or KRX) in LB, 2XYT or Studier's induction media did not succeed (data not shown).

Purification of the recombinant CphAII

The enzyme was purified from the soluble fraction of *E. coli* GI724 (cytoplasmic and periplasmic fractions) after 20 h of growth by heat-denaturation of contaminant *E. coli* proteins followed by two steps of nickel-affinity chromatography. Purified CphAII migrates as a 35-kDa protein on SDS-PAGE, which corresponds well to the calculated molecular mass of the mature CphAII protein fused to the polyhistidine-tag. Western blot analysis with anti-His-tag antibodies confirmed the presence of the His-tag in the ~35 kDa band (data not shown). Moreover, in-gel tryptic digestion of the ~35 kDa band was performed. Mass spectrometric (MS) analysis of the trypsin-digested peptides confirmed that the purified protein was CphAII. The purified protein was confirmed to be in a monomeric form of about 35 kDa by comparing its retention volume to those of standard proteins upon gel filtration chromatography.

The N-terminal sequence predicted by the SignalP 3.0 program (Bendtsen et al. 2004) is MTLKK; however, the experimental N-terminal sequencing resulted in an unambiguous N-terminal sequence of LIISTAF. The mature CphAII protein produced in *E. coli* had thus eight additional amino-acids at the N-terminal when compared to the predicted mature enzyme (Fig. 1b). Moreover, the mass of the protein verified by electrospray mass spectrometry corresponded within experimental error to the calculated mass for CphAII beginning at LIISTAF and in fusion with a LEHHHHH-peptide (His-tag) on the C-terminal (35,101 vs. 35,099 Da).

Recombinant CphAII produced in *E. coli* is able to bind zinc ions

After extensive dialysis against a 50-mM sodium phosphate buffer pH 8.0 containing less than 0.4 μM free zinc, ICP/MS showed that the CphAII protein produced in *E. coli* contained one zinc ion per molecule. After extensive dialysis against a 50-mM sodium phosphate buffer pH 8.0 containing 100 μM zinc, CphAII bound a second zinc ion. As isolated, CphAII contained a His-tag which could not be removed. The absence of influence of a His-tag on the number of bound zinc ions as determined by ICP/MS has already been demonstrated with two others proteins of the superfamily, ZiPD and the cytosolic oxygen reductase from *E. coli* (Vogel et al. 2002). Nevertheless, to verify that the His-tag had no influence on the number of zinc ions bound by CphAII, the protein was also produced from the original pLex vector and purified without the tag. The yield of this purification was very low and the quantity of pure CphAII obtained was only sufficient to carry out the same zinc content measurements which confirmed the data obtained with the His-tagged enzyme (Table 1). Other metallic ions such as cobalt, copper, manganese, iron and nickel were not found.

CphAII possesses a phosphodiesterase activity

Based on the sequence identity with the CphA and SPM-1 MBLs, we probed CphAII first for β-lactamase activity using carbapenem, penicillin and cephalosporin substrates.

Table 1 Determination of the Zn/enzyme content by ICP/MS

$[Zn^{2+}]$ in the dialysis buffer	$[Zn^{2+}]$ ($\mu g\ l^{-1}$)	$[Zn^{2+}]$ (μM)	Zn/CphAII ratio
<0.4 μM			
Dialyzed CphAII sample	981	15	
Dialysis buffer	<10	<0.15	
→ CphAII (15 μM)	=981–10	14.9	1
100 μM			
Dialyzed CphAII sample	8,032	124	
Dialysis buffer	6,147	95	
→ CphAII (15 μM)	=8,032–6,147	29	1.9

No activity toward these substrates could be detected at 21°C. At 85°C, these antibiotic compounds were too unstable to perform accurate kinetics measurements. However, the addition of the enzyme did not increase the hydrolysis rate.

Also, CphAII was probed for enzymatic activity (phosphatase, phosphodiesterase, nuclease, esterase, protease, dehydrogenase, oxidase and sulfatase) using several general enzymatic assays. Particularly, compounds modified by other enzymes of the MBL superfamily were tested including glyoxalase II, arylsulfatase, phosphatase and phosphodiesterase substrates. Two phosphodiester substrates were identified: TpNPP (thymidine 5′-monophosphate *p*-nitrophenyl ester) and bpNPP (bis-para-nitrophenyl-phosphate). bpNPP was a much better substrate. It was cleaved into *p*-nitrophenol and *p*-nitrophenyl phosphate. The latter product was also tested as substrate and was not further hydrolyzed by the enzyme. This enzyme is therefore a phosphodiesterase and does not act on phosphomonoesters.

Various pH and buffers were assayed for the stability and the activity. The stability and the phosphodiesterase activity were maximal in 50 mM sodium phosphate buffer pH 8.0, containing 250 mM NaCl. The enzyme was stable at 50°C for more than 24 h and at least 3 h at 90°C.

The phosphate buffer, thanks to its low thermal coefficient, is ideal to work at high temperature (its pH only slightly varies with temperature when compared to organic buffers such as Tris). In this buffer, the recombinant CphAII protein showed activity toward the generic phosphodiesterase substrate bpNPP, with a k_{cat} value of 0.02 s^{-1} and a K_m value of 6 mM when assayed at 21°C (Fig. 2). The CphAII phosphodiesterase activity increased at higher temperatures, reaching a k_{cat} of 1 s^{-1} with a K_m of 5 mM at 85°C (Fig. 2).

As ZiPD (Vogel et al. 2002), CphAII hydrolysed TpNPP, another general phosphodiesterase substrate, at a significantly lower rate than bpNPP. Indeed, the yellow color resulting from the presence of *p*-nitrophenol only appeared after an overnight incubation with the enzyme at 21°C. At 85°C, CphAII hydrolysed TpNPP with a k_{cat} of 0.2 s^{-1} and a K_m of 14 mM (Fig. 2).

CphAII exhibited neither phosphodiesterase activity with the cyclic nucleotides tested (2′,3′-cCMP, 3′,5′-cAMP or 3′,5′-cGMP), nor nuclease activity with various substrates (single- or double-stranded DNA, RNA), nor phospholipase activity towards phosphatidylcholine. Also, no cleavage of pyrophosphate, AMP, ADP or ATP was detected after incubation with CphAII. At 85°C, CphAII showed a very low but detectable activity versus pNPPC (phosphodiesterase/phospholipase C substrate). The yellow color due to the liberation of the *p*-nitrophenol only appeared after 4 h of incubation with 5 μM CphAII.

The activity of CphAII is metal-dependent

At 85°C, zinc and cobalt ions showed activating effects on the activity of CphAII versus bpNPP. The phosphodiesterase activity increased upon addition of zinc or cobalt ions to the mono-zinc form. The determined K_{D2} values for the binding of the second metal ion were 16 and 1.3 μM for zinc and cobalt, respectively. Saturating concentrations of the metal ions resulted in 1.4- and 1.9-fold activity increases with zinc and cobalt, respectively.

CphAII was more than 99% inactivated by the loss of the metal after 1 h incubation with 10 mM EDTA. After subsequent removal of EDTA by three successive cycles of dilution/concentration with buffer A1 beforehand treated with CHELEX (Sigma), the addition of 50 μM Zn^{2+} to the apoenzyme of CphAII did only restore 8% of the initial activity versus 30% with 50 μM Co^{2+}.

Structural studies

The room temperature far-UV CD spectrum of CphAII exhibited two local minima at 208 and 218 nm. CphAII is primarily an α/β protein: the helical, β-sheets, turns and unordered contents predicted using the CDSSTR simulation program were 30, 22, 20 and 28%, respectively (Fig. 3). These data are similar to the values previously obtained using the same simulation program for the *Bacillus cereus* MBL (BcII) (30% α-helix, 20% β-strand, 19% turns and 31% disordered) (Jacquin et al. 2009) and

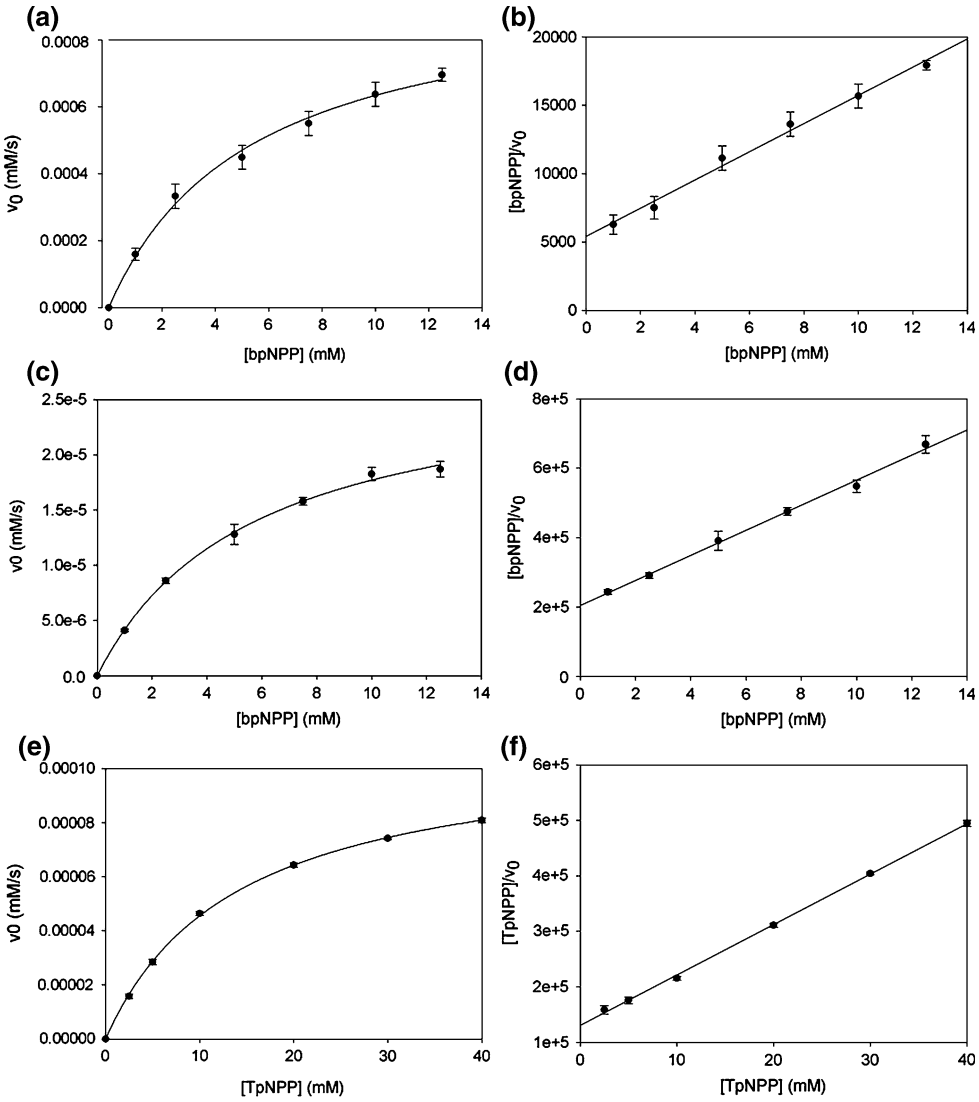

Fig. 2 **a**, **c** and **e** The initial rate (v_0) versus substrate concentration [bpNPP (**a**, **c**) and TpNPP (**e**)], at 21°C (**c**) and 85°C (**a**, **e**). The reaction conditions were as described in "Experimental". Measurements were performed in triplicate. Experimental data were fitted to the Henri–Michaelis–Menten equation by non-linear regression analysis with the help of the SigmaPlot software. **b**, **d** and **f** Hanes linearization of the kinetic data presented in **a**, **c** and **e**, respectively

with the X-ray data available for CphA (i.e., 29% α-helix and 24% β-strand, calculated in the Research Collaboratory for Structural Bioinformatics Protein Data Bank, using the 1X8G Protein Data Bank structure).

We attempted to obtain values for the apparent melting temperatures (T_m) for the protein by measuring the ellipticity at 222 nm upon heating of the samples from 25° to 95°C. The CphAII protein showed a gradual but steady loss of signal, and post-transition baselines could not be established (T_m values > 95°C). In the presence of 10 mM EDTA, the T_m value was also above 95°C.

Discussion

In this study, we developed an overexpression system in *E. coli* for the *A. aeolicus* CphAII protein, as well as a simple protocol based on the presence of a His-tag for purification of the recombinant enzyme. A first characterization of the enzyme was performed.

Sequence alignment shows that CphAII belongs to the MBL superfamily (Bebrone 2007; Daiyasu et al. 2001; Aravind 1999; Neuwald et al. 1997), which suggests that CphAII possesses two metal-binding sites. Indeed, CphAII contains the five conserved motifs of the MBL superfamily, namely Asp84, His116-Xaa-His118-Xaa-Asp120-His121, His 196, Asp221 and His263. In the structurally characterized members of the superfamily and with the exception of Asp84, all these conserved residues participate in metal binding. We have shown here that CphAII purifies as a mono-zinc form from the soluble fraction of *E. coli*. Its activity is metal-dependent and both zinc and cobalt ions have a positive effect on the activity of CphAII. So, we cannot exclude that the cobalt-CphAII form could be

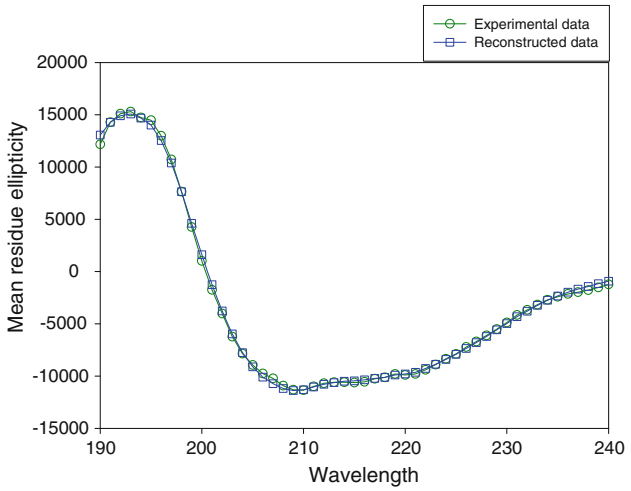

Fig. 3 Far-UV CD spectrum of CphAII. Deconvolution of the CD spectrum was performed using the CDSSTR program found in DICHROWEB (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml)

naturally produced in the hyperthermophile *A. aeolicus*. The enzyme exhibits a higher activity after addition of cobalt to the mono-zinc form, which probably yields the Zn/Co hybrid. This is often found with zinc-containing proteins of the MBL superfamily. For example, in the subclass B1 *Bacillus cereus* MBL (BcII), cobalt is also more efficient than zinc for binding to the second site (Orellano et al. 1998). MBLs are able to function with cobalt (Lisa et al. 2010; Hawk et al. 2009) and the cobalt-substituted MBL from *Bacillus cereus* (BcII) is even a more efficient catalyst than the zinc native enzyme for the hydrolysis of some β-lactam antibiotics (Badarau et al. 2007). Also, the most active form of the human glyoxalase II, another member of the superfamily, is the cobalt-substituted form (Limphong et al. 2009).

The predicted percentage of helices and strands obtained using the deconvolution of the far-UV CD spectrum is in agreement with the content in α and β structures of MBLs. Moreover, molecular modeling results confirmed the high probability of the $\alpha\beta\beta\alpha$ fold (data not shown).

Despite 28 and 25% identities with the subclass B2 CphA and the subclass B1 SPM-1 MBLs, respectively, CphAII is not a β-lactamase but possesses a weak phosphodiesterase activity. The best substrate found here was the artificial bpNPP phosphodiester, while TpNPP was a significantly poorer substrate. Expectedly for an enzyme from a hyperthermophilic organism, the activity of CphAII is optimal at 85°C. At the optimum, the kinetic k_{cat} and K_m constants of CphAII are similar to those previously determined for another member of the MBL superfamily, the *E. coli* K12 PhnP protein which is a phosphodiesterase of the carbon-phosphorus lyase pathway for phosphonate degradation (Podzelinska et al. 2009) (Table 2). Although the k_{cat}/K_m value of CphAII for bpNPP (200 M^{-1} s^{-1}) is

Table 2 Comparison of the kinetic parameters of several characterized phosphodiesterases with those of CphAII

	Added metal	bpNPP			TpNPP			Temp (°C).	Reference
		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})		
CphAII	–	0.02	6	3	–	–	–	21	This work
CphAII	–	1	5	200	0.2	14	14.3	85	This work
E. coli PhnP	(1 mM Mn^{2+})	1.19	2.9	410	0.08	15	5.3	25	Podzelinska et al. (2009)
E. coli PhnP	(100 μM Zn^{2+})	0.03	0.68	44	–	–	–	25	Podzelinska et al. (2009)
Arabidopsis thaliana tRNaseZ	–	7.4	8.5	870	–	–	–	37	Späth et al. (2007)
E. coli ZiPD	–	59	4	15,000	9	3	3,000	22	Vogel et al. (2002)
Methanococcus jannaschii MJ0936	(1 mM Ni^{2+})	105.7	0.15	710,000	3.26	0.035	94,000	70	Chen et al. (2004)
Restriction enzyme *BfiI*	–	–	–	4.2	–	–	–	25	Sapranauskas et al. (2000)
Endonuclease *Nuc*	–	0.12	8.5	14	–	–	–	37	Zhao et al. (1997)

much lower than that of the ZiPD/ElaC phosphodiesterase from *E.coli* (15,000 M^{-1} s^{-1}) (Vogel et al. 2002) (Table 2), it is one and two orders of magnitude higher those of *Nuc* (14 M^{-1} s^{-1}, Zhao et al. 1997) and *Bfi*I (4.2 M^{-1} s^{-1}, Sapranauskas et al. 2000) nucleases, respectively. Note that ZiPD/ElaC is a binuclear zinc tRNAse Z which also belongs to the MBL superfamily (Schilling et al. 2005; Vogel et al. 2002).

In vitro, CphAII demonstrates metal-dependent phosphodiesterase activity versus artificial bpNPP and TpNPP substrates. A very low but detectable activity versus pNPPC (phosphodiesterase/phospholipase C chromogenic substrate) is also observed. In our studies, CphAII showed no nuclease activity against single- or double-stranded DNA or RNA. Moreover, no phosphohydrolase activity was found toward 2′,3′- and 3′,5′-cyclic nucleotides or phosphatidylcholine. The sequence of CphAII does not present the tRNAse Z-specific insertion of about 50 kDa located between His196 and Asp221 which is required for tRNA binding and pre-tRNA processing (Ishii et al. 2005; Schilling et al. 2005). With the exception of conserved motifs in the superfamily, CphAII does not present any sequence similarity to ZiPD/ElaC. Also, CphAII does not contain the β-CASP region specific to members of the MBL superfamily acting on nucleic acid substrates and involved in DNA repair and RNA processing (Callebaut et al. 2002). At the present time, the natural substrates(s) of CphAII remain(s) undetermined. It is, however, quite likely that this activity is highly specific. We are trying now to obtain the three-dimensional structure of CphAII in order to discuss these structural results in the context of its mechanism of action and biological function as a phosphodiesterase.

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